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Award Number: W81XWH-06-1-0292

TITLE: Role of Obesity in Prostate Cancer Development

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REPORT DATE: April 2011

TYPE OF REPORT: Final Addendum

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-04-2011	Final Addendum	15 FEB 2009 - 31 MAR 2011

4. TITLE AND SUBTITLE

Role of Obesity in Prostate Cancer Development

5a. CONTRACT NUMBER

5b. GRANT NUMBER

W81XWH-06-1-0292

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Minnesota
Minneapolis, MN 55455

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Abstract on next page.

15. SUBJECT TERMS

TRAMP mice, TRAMP-C2 cells, obesity, prostate cancer

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	29	19b. TELEPHONE NUMBER *(include area code)*

14. ABSTRACT

Prospective epidemiological studies suggest that obesity increases the risk for prostate cancer. Also, mortality from prostate cancer is increased with elevated body weights and several recent studies have indicated that obesity was reported to be associated with higher prostate cancer grade at diagnosis. However, it is difficult in human studies to adequately assess effects of body weight and/or the effects of body weight change at specific ages on prostate cancer given the long life span. Recent introduction of the TRAMP (transgenic adenocarcinoma mouse prostate) mouse provides a model that shares many characteristics with human prostate cancer. The initial goal of this proposal was to determine the effect of obesity induced at different ages on the development of prostate cancer in TRAMP mice. Obesity was induced by injections with gold-thioglucose (GTG) at either 6, 16 or 26 weeks of age. Body weight was monitored and the mice were followed until 46 weeks of age. Unfortunately, there was an unexpectedly high mortality rate in TRAMP mice in response to the GTG injections. This resulted in a limited number of mice available to follow. However, the results we did obtain indicated little effect of obesity regardless of age of onset on prostate cancer development in this model. A second experiment was conducted using a prostate cancer cell line developed from a TRAMP mouse tumor, TRAMP-C2. Since this cell line was developed from a mouse on the C57BL6 background it can be inoculated into wild-type mice and tumor growth can then be monitored. C57BL6 male mice were fed a high fat diet and then divided by body weight into Obesity-Prone, Overweight and Obesity-Resistant groups with an additional group fed a low-fat diet. Mice were inoculated with the cell line and tumor growth followed. The high-fat diet per se affected tumor weight and size. Interestingly, genital-urinary and prostate weights were highest in the Obesity-Prone mice. A third study (partially funded by another agency) used the dietary-induced obesity protocol in TRAMP mice. Overweight and Obesity-Prone mice had higher incidence of moderately or poorly differentiated adenocarcinomas compared to Obesity-Resistant and Low-Fat mice. Synaptophysin protein expression was measured in tumors as an indicator of neuroendocrine origin. All mice had similar percentage of tumors positive for synaptophysin, 35%. However, synaptophysin negative tumors were more apt to be high grade and poorly differentiated in the mice fed the high fat diet regardless of body weight status. In conclusion these findings are consistent with human data suggesting that high body weight is associated with more aggressive prostate cancer at diagnosis but diet may also play a role.

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INTRODUCTION:

A number of epidemiological studies indicate that increased body weight plays a role in the development of prostate cancer [1-9]. Although, not all studies have found obesity to be associated with increased risk of prostate cancer, Bergström *et al* concluded that based on obtained relative risk values 5,000 new cases of prostate cancer per year in Europe could be attributed to obesity [10]. In addition, mortality from prostate cancer is increased with elevated body weights [11], and obesity was reported to be associated with higher prostate cancer grade at diagnosis, as well as with higher recurrence rates [12]. The potential role of body weight in the development and progression of prostate cancer is of interest given that the incidence of overweight/obesity is increasing throughout the world, and the potential for lifestyle changes to alter body weight status. Interestingly, since we originally submitted this proposal an increasing number of publications have addressed the issue of obesity and its association with the development and prognosis of prostate cancer [13-17]. It has also been reported that prostate cancer cells from men with higher BMI (body mass index) are associated with altered levels of genes involved in lipid and cholesterol metabolism and even normal prostate tissue might be affected [18].

The initial goal of the present proposal was to evaluate the effects of obesity initiated at different ages on prostate cancer development using the TRAMP mouse model. Obesity was to be induced at different ages (6, 16 and 26 weeks of age) using gold-thioglucose (GTG) injections. Results of that study will be presented. However, there were a number of problems with this protocol primarily a very high mortality rate of TRAMP mice receiving GTG which was not anticipated based on the literature as well as preliminary studies we conducted using wild-type C57BL6 mice. As a result this aspect of the study was suspended and a different approach was implemented using a cell line developed from a TRAMP mouse tumor, TRAMP-C2. These cells were implanted into mice with different body weights following consumption of a high fat diet. We also conducted a diet-induced obesity study which was mostly funded by American Institute for Cancer Research (AICR). However, we have compared the results to those obtained for the GTG study in particular with respect to the analyses of prostate cancer tissue for the expression of the protein synaptophysin as an indicator of neuroendocrine origin of the tumors.

BODY:

Our first study was to determine the effect of obesity initiated at specific ages on prostate cancer in the TRAMP mouse model. It was reported many years ago that a single injection with GTG resulted mice gaining weight and becoming obese [19]. Not all mice responded and it is unclear why as when they are re-injected with the same dose of GTG they then develop obesity. This is an important observation as it indicates that the initial lack of response is not due to resistance to GTG. Shortly after leptin was identified, GTG-induced obesity was reported to increase plasma leptin as identified by immunoblot; and leptin mRNA expression in adipose tissue was elevated compared to lean animals [20]. More recently when leptin levels were assessed by commercially available radioimmunoassay kits, GTG obese mice were found to have serum leptin levels two-fold higher than in control mice [21;22]. Body weight gain without consumption of a high-fat diet is obtained, although food intake is initially increased in GTG-treated mice [21;23]. Body weights eventually plateau and caloric intakes are appropriate for body weights. There is no age-sensitive time-point at which GTG needs to be administered in order to produce the effect on body weight. For example, reported results include mice injected as young as 3-4 weeks of age [24-27]. In other studies mice were anywhere from 8 to 20 weeks of age [19;21;23;28;29]. Also

different GTG doses over a range from 0.3-2.0 mg/g body weight have been used. In our own study in female nude mice we found that a dose of 0.5 mg/g resulted in a high mortality rate therefore we decided to undertake a preliminary study in male mice prior to injecting the TRAMP mice with GTG.

Pilot study 1:

GTG was injected into 14 male wild-type mice at a dose of 0.5 mg/g body weight. A control group of six male wild-type mice was injected with the same volume of PBS. One mouse became ill after receiving GTG and was euthanized. Mice ranged in age from 8 to 23 weeks and were followed for ten weeks to monitor body weight changes and general body condition. Fifty-four percent (7 out of 13) of the mice became obese. The GTG obese cohort gained significantly more weight than either the GTG non-obese or the control mice (Figure 1). There was no significant difference in weight gain between the non-obese and the PBS injected mice. In comparison to our earlier study in female mice the male mice tolerated the 0.5 dose well.

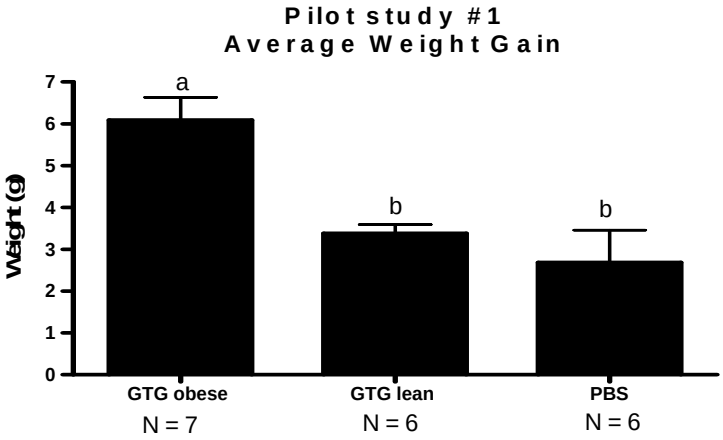


Figure 1: 10-week weight gain during pilot study 1. ANOVA P = 0.001; GTG obese versus GTG lean P < 0.01; GTG obese versus PBS P < 0.01; GTG lean versus PBS P > 0.05.

Pilot study 2:

Since the 0.5 dose was well tolerated and in an effort to increase the percentage of mice that become obese a dose of 0.8 mg/g body weight of GTG was injected into 12 male wild-type mice. A control group was made up of five male wild-type mice injected with PBS. Four mice became ill after receiving GTG and they were euthanized. The mice, ranging in initial age from 11 to 13 weeks, were then followed for 10 weeks to monitor body weight changes and general body condition. Sixty-three percent (5 out of 8) became obese. The GTG obese cohort gained significantly more weight than either the GTG non-obese or the PBS-injected mice (Figure 2). There was no significant difference in the 10-week weight gain between the non-obese and the control mice.

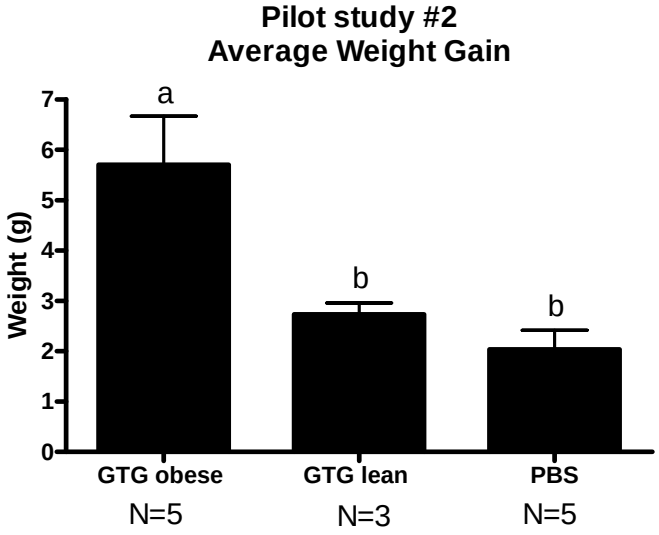


Figure 2: 10-week weight gain during pilot study 2. ANOVA P = 0.0077; GTG obese versus GTG non-obese P < 0.05; GTG obese versus PBS P < 0.01; GTG lean versus PBS P > 0.05.

Study of TRAMP mice injected with GTG at three different ages

General Methods: All mice had *ad libitum* access to purified AIN-93M diet and water. Following the pilot studies we determined that the 0.8 dose would be used. Body weights were recorded weekly and at which time mice were palpated for tumors. Mice that received GTG were categorized as obese or non-obese based on weight gain relative to the PBS control mice. Serum samples were collected from the retro-orbital sinus at baseline and every 5 weeks until a tumor was palpated. Following tumor palpation, serum was collected every 3 weeks until study termination. Data are presented as mean ± SE.

Survival: To our surprise the TRAMP mice did not tolerate the GTG as well as anticipated and several adjustments were made to the protocol in attempts to improve survival. The dose of 0.8 mg/g was best tolerated in the mice injected at 26 weeks of age; this cohort had the highest survival at 42%. Mice in the 6- and 16-week cohorts had a much lower rate of survival at this dose, 0 and 13%, respectively. Lowering the dose to 0.5 mg/g increased survival of 6 weeks of age mice to 23%, but only 9% survived at this dose when injected at 16 weeks of age. A summary of the survival data is presented in Table 1.

Table 1. Percent Surviving after GTG Injection		
	0.8 mg/g	**0.5 mg/g**
6-week	0%	23%
16-week	13%	9%
26-week	42%	not done

Obesity rate, weight gain and fat pad weights: After receiving GTG injections, as expected, some mice rapidly gained weight and were characterized as GTG-obese, while others did not and were named as GTG-lean. The percent that became obese was identical for the 6- and 26-week cohort, 57%. In the 16-week cohort, only 33% became obese. Body weight curves for the three cohorts are shown in Figure 3.

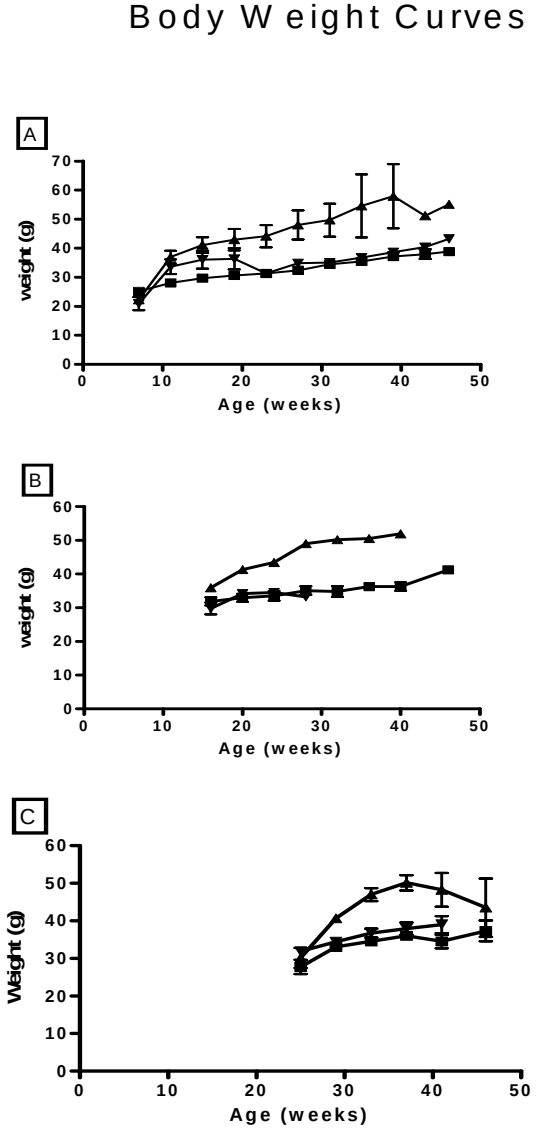


Figure 3. Body weight curves of TRAMP mice. **Panel A** mice injected at 6 weeks of age, GTG-obese n = 1-4; GTG-lean n= 1-3 and PBS n = 2-15 dependent upon age; **Panel B** mice injected at 16 weeks of age, GTG-obese n = 1; GTG-lean = 1-2; PBS n = 4-13 dependent upon age; **Panel C** mice injected at 26 weeks of age, GTG-obese n=2-8; GTG-lean n = 1-6; PBS n = 2.-12 dependent upon age. ▲ = GTG-obese;▼ = GTG-lean; ■ = PBS control. For all three cohorts overall ANOVA $p<0.001$ GTG-obese versus PBS.

The GTG-lean mice and PBS control mice had similar body weights over the course of the study for all three age groups. The actual weight gains are provided in Figure 4 where it can be seen that the GTG-lean mice gained a similar amount of weight as did control mice given only saline injections. As expected GTG-obese mice gained significantly more weight than did the two lean groups. Figure 5 presents fat pad weights which were similar in the GTG-lean and control mice and were significantly lower compared to the GTG obese mice in each age cohort.

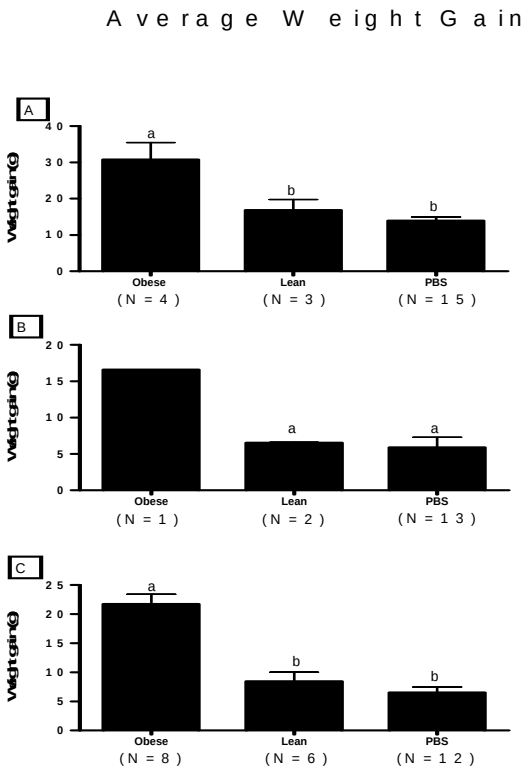


Figure 4. Average weight gain of TRAMP mice in the 6 week (Panel A), 16 week (Panel B), and 26 week (Panel C) cohorts. For both the 6 and 26 week cohorts ANOVA $p<0.0001$ and columns with different superscripts significantly different from each other. For the 16 week cohort t test between the two lean groups was not significantly different.

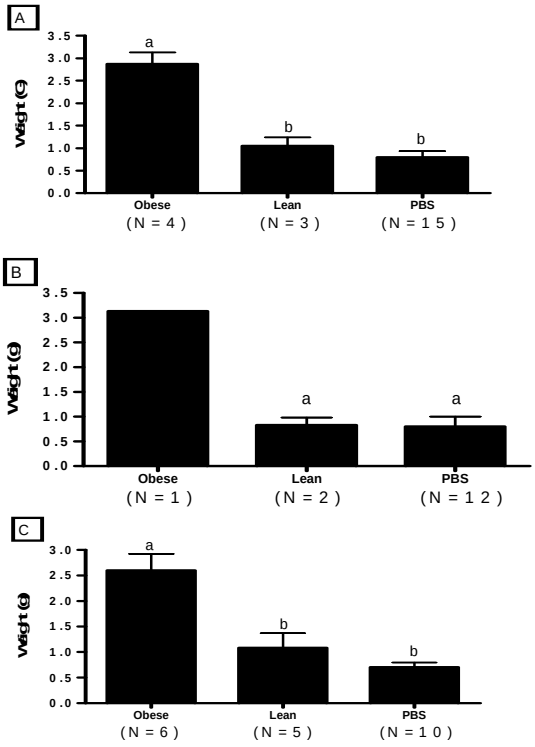


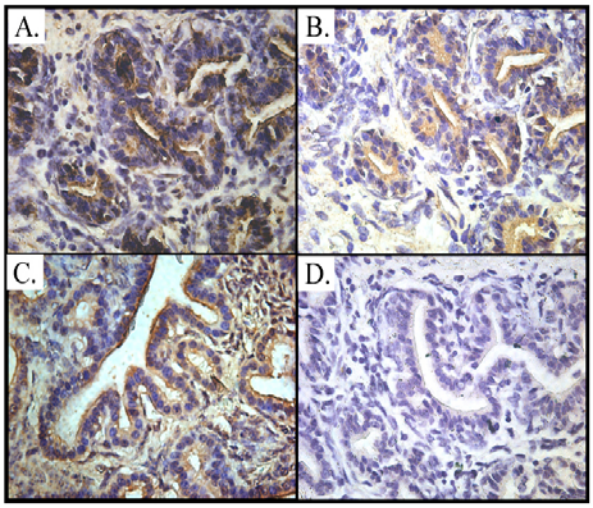
Figure 5. Average fat pad weights (sum of epididymal and retroperitoneal) for TRAMP mice in the 6 week (Panel A), 16 week (Panel B) and 26 week (Panel C) cohorts. For 6 and 26 week cohorts ANOVA $p<0.001$, columns with different superscripts are significantly different. For 16 week cohort t test not significant between the lean and PBS groups.

Table 2. End point comparisons for TRAMP male mice [§]

GTG-6	Final body weight (g)	Age at tumor palpation (weeks)	Age at death (weeks)	GU weight (g)	Tumor differentiation			Percent with metastasis
					Well	Moderate	Poor	
Obese (N=4)	51.78 ± 5.30[a]	27.67 ± 4.91 (N=3)	35.75 ± 4.59	6.23 ± 1.40	50%	25%	25%	25%
Lean (N=3)	36.07 ± 3.75[b]	22.0 ± 3.51	28.33 ± 8.84	6.30 ± 1.96	33%	0	67%	67%
PBS (N=15)	36.92 ± 1.33[b]	29.36 ± 1.61 (n=14)	38.33 ± 1.92	7.84 ± 0.84	60%	7%	33%	47%
GTG-16	**Final body weight (g)**	**Age at tumor palpation (weeks)**	**Age at death (weeks)**	**GU weight (g)**	**Tumor differentiation**			**Percent with metastasis**
					Well	Moderate	Poor	
Obese (N=1)	51.9	30	40	9.42		100%		0%
Lean (N=2)	33.2 ± 1.0	25 (N=1)	24 ± 4	3.87 ± 2.4			100%	100%
PBS (N=13)	35.5 ± 1.7	26.5 ± 1.4 (N=12)	35.6 ± 2.5	7.3 ± 0.8	54%	8%	38%	46%
GTG-26	**Final body weight (g)**	**Age at tumor palpation (weeks)**	**Age at death (weeks)**	**GU weight (g)**	**Tumor differentiation**			**Percent with metastasis**
					Well	Moderate	Poor	
Obese (N=8)	48.61 ± 2.60[a]	33.38 ± 0.94	42.13 ± 1.22	9.10 ± 1.54 (N=6)	50%	50%	0%	13%
*Lean (N=6)	39.08 ± 2.46[b]	33.5 ± 1.09	43.0 ± 1.67	8.90 ± 1.45 (N=5)	67%	17%	16%	17%
PBS (N=12)	36.65 ± 1.63[b]	32.3 ± 1.1	41.3 ± 1.4	8.16 ± 1.0 (N=11)	58%	8%	33%	33%

§ columns with different letters indicate a significant difference among the groups
*pathology report not received for one mouse in this group

Prostate cancer results: A summary of the results for prostate cancer development for the cohorts is presented in Table 2. In all three cohorts final body weights were significantly higher for the GTG-obese mice compared to the GTG-lean and PBS control mice. For the other determinations there were no significant differences which may partially be attributable to the small sample sizes in GTG-obese and GTG-lean groups. However, a few interesting observations were made. For example, the GTG-obese mice injected at 6 weeks of age had a delay in tumor detection compared to the GTG-lean mice and a delayed age at death. Genital-urinary tract (GUT) weight was not impacted by body weight. Tumor differentiation was improved and metastases rate was reduced in GTG-obese mice compared to the PBS control mice. Due to the poor survival rate for the mice injected at 16 weeks of age it was not possible to make conclusions for this group. For the mice injected at 26 weeks of age, age of tumor detection, age at death and GUT weights were similar in all three groups. There was however, a trend for GTG-obese mice to have an improved tumor differentiation profile compared to both lean groups and to have a reduced metastasis rate compared to the control saline injected mice. As indicated above serum samples were obtained from the mice. We attempted to evaluate the samples for testosterone and estradiol levels in relationship to obesity status and in relationship to age of tumor detection. However, in general these results did not provide any consistent findings.



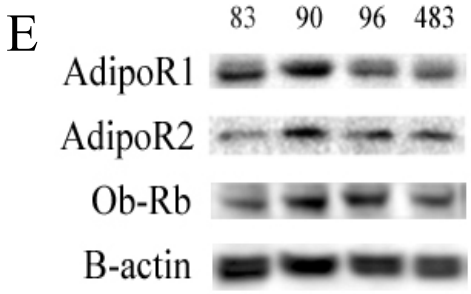


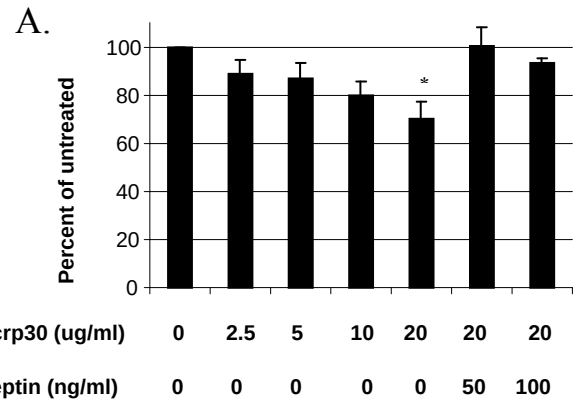
Figure 6. Expression of Acrp30 and leptin receptors from TRAMP prostate tumor tissue. Immunohistochemistry of A) AdipoR1, B) AdipoR2, C) Ob-Rb and D) control with goat serum instead of primary antibody. The presence of reddish brown color indicates a positive reaction for the specific primary antibodies. Arrows indicate areas of positive staining for each antibody. Hematoxylin was utilized for the blue counter staining of the nuclei. E) Western blots of AdipoR1, AdipoR2 and Ob-R from TRAMP prostate tumor tissues from four different TRAMP animals. The individual animal numbers at the top of the blots. The antibodies used are shown along the left hand side.

Expression of AdipoR1, AdipoR2 and Ob-Rb in tumor tissue from mouse prostate cancer: Because of the increasing interest in body weight and how it might affect prostate cancer and due to the findings of in vitro studies with leptin and adiponectin [30-34] which are both produced in adipose tissue we investigated whether tumors from TRAMP mice express the two receptors for Acrp30, AdipoR1 and AdipoR2 and the signaling form of the leptin receptor, Ob-Rb. Tumors from four different mice were examined using immunohistochemistry and Western blot analysis. Representative staining from the immunohistochemistry is shown in Figure 6. Expression of AdipoR1 was found in all prostate tumor tissue from TRAMP mice and was primarily located in epithelial cells on the apical region (Fig 6A). In samples from all mice AdipoR2 (Fig 6B) was present primarily in the same areas as AdipoR1 but the staining was less intense. Ob-Rb (Fig 6C) was expressed throughout the samples. Control staining with goat serum instead of primary antibody was negative (Fig 6D). Western blot analysis of frozen tissue from the same mice was also performed and expression of AdipoR1, AdipoR2 and leptin receptor was found in all prostate tumor tissues examined (Fig 6E).

Study using TRAMP-C2 Cells

Because of the difficulties with GTG injections described above we decided to take a different approach to evaluate the effect of obesity on prostate tumor development. We utilized a diet-induced obesity regimen which we had used previously in studying mammary tumor development in transgenic mice as well as in a xenograft study [35-38]. C57BL6 mice are particularly susceptible to become obese when fed a high-fat diet. Furthermore, use of this strain provides the unique opportunity to evaluate prostate cancer cell ability to develop tumors in mice fed the same diet but with different body weights. Previous studies in our lab and in other labs have shown that although most C57BL6 mice fed a high-fat diet will gain weight and become overweight or obese, some mice will stay in the body weight range of low-fat fed mice [35;39]. This occurrence provides the opportunity to compare mice of the same body weight consuming diets of different composition as well as to compare mice fed the same diet but with different body weights. C57BL6 mice have a normal immune system and may be implanted with the syngeneic Tramp-C2 tumor cells which will cause tumors to develop [40;41]. Using this model we investigated the influence of a high fat diet on TRAMP-C2 cell growth in vivo. We also determined the effects of differences in weight by comparing obesity resistant vs obesity prone mice for TRAMP-C2 tumor growth. The use of this cell line provides a straight forward approach which is much simpler than using athymic mice in xenograft studies of human prostate cancer cells as the mice do not need to be maintained in an ultraclean environment. Also it is fortuitous to be able to use C57BL6 mice as these mice readily develop dietary-induced obesity.

In vitro study: Prior to the mouse study we did in vitro studies to evaluate the effects of the addition of leptin and adiponectin to the TRAMP-C2 cells. These results are presented in Figure 7.



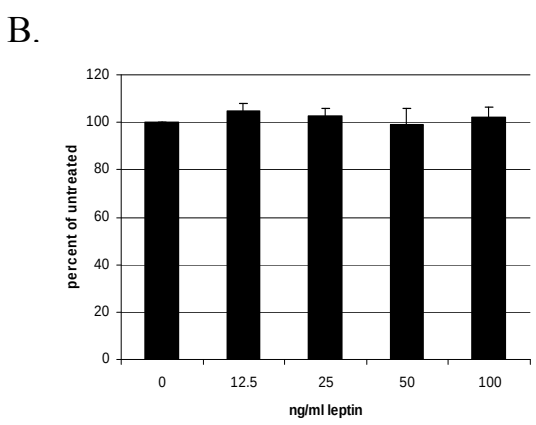


Figure 7. Proliferation of TRAMP-C2 cells 48 hours after treatment with Acrp30 (adiponectin), leptin or both. Cell proliferation as a percent is shown along the y-axis. Cells in serum-free media were considered to be 100%. A) The concentrations of Acrp30 and leptin are shown along the x-axis. Bars represent standard error of the mean from four different experiments and asterisks indicate significant difference from untreated. (ANOVA p=0.0393). B) Cell proliferation of the TRAMP-C2 cells in response to increasing levels of leptin (ng/ml). The concentration of leptin is shown along the x-axis. Bars represent standard error of the mean from three different experiments. There was no effect of leptin alone on TRAMP-C2 cell proliferation.

It can be seen that when the TRAMP-C2 cells were treated with Acrp30/adiponectin in the physiological range of 2.5-20 μg/ml there was a dose-related reduction in proliferation of the TRAMP-C2 cells after 48 hours (Fig 7A). The difference in proliferation was statistically significant at the Acrp30 concentration of 20 μg/ml compared to the untreated cells. The cells had 70% proliferation compared to the untreated controls. In addition, cells were treated with a combination of Acrp30 and leptin. We found that the addition of leptin blocked the ability of Acrp30 to inhibit proliferation. However, figure 7B shows that treatment of the cells with leptin alone in its physiological range for 48 hours did not result in a statistically significant change in cell proliferation. These data strengthen the possibility that prostate cancer proliferation can be inhibited by Acrp30 and that leptin can block this protective effect. This would provide an explanation for how obesity could impact prostate cancer cell development and/or progession because in the obese state the high levels of serum leptin and low levels of adiponectin would be permissive for cell proliferation.

In vivo experiments with TRAMP-C2 cells: We obtained C57BL6 male mice (n=160) from Jackson Laboratory, Bar Harbor ME in groups of 40. Upon arrival at 4 weeks of age mice were maintained on AIN-93M diet [38;42]. At 6 weeks of age 120 mice were switched AIN-93M-High-Fat diet [38]. At 20 weeks of age mice were implanted with the TRAMP-C2 cells ($3x10^6$) in the left flank. Mice were weighed and palpated for tumors over the next 10 weeks. At the termination of the study a blood sample was obtained and the tumors were removed, measured and weighed and then processed for histopathology. Epididymal and retroperitoneal fat pads were removed as well as genitourinary tracts and prostates.

Weight gain and visceral fat pad weights in low-fat fed and high-fat fed mice: Figure 8A shows the weights of the 4 different groups of mice at 25 weeks of age (this age was used to avoid potential problems as the tumors grew). As expected, the mice fed a high-fat diet could be divided into three weight groupings, obesity resistant, overweight and obesity-prone [35]. Five of the high-fat fed mice had to be removed from the study due to non-study related illnesses. All 40 of the low-fat fed mice finished the study. The ANOVA for the mouse weights was $P<0.0001$. All groups were significantly different from each other ($P<0.001$). The low-fat fed mice weighed on average 35.4 grams, the high-fat fed obesity resistant mice averaged 33.8 grams which was significantly different from the low-fat fed mice. The high-fat fed overweight mice averaged 37.6 grams and the high-fat fed obesity-prone mice weighed an average of 41.1 grams. We also weighed the visceral fat pads from the mice (Fig 8B) when they were sacrificed. Similar results were found as compared to the body weights. The ANOVA for the visceral fat pads was $P<0.0001$. The lightest visceral fat pads were from the low-fat fed mice (1.78 grams) and the high-fat fed obesity resistant mice (1.73 grams) and these were not significantly different from each other. The visceral fat pads of the high-fat fed overweight mice averaged 2.16 grams and the visceral fat pads from the high-fat fed obesity-prone mice weighed an average of 2.49 grams and were significantly different from each other.

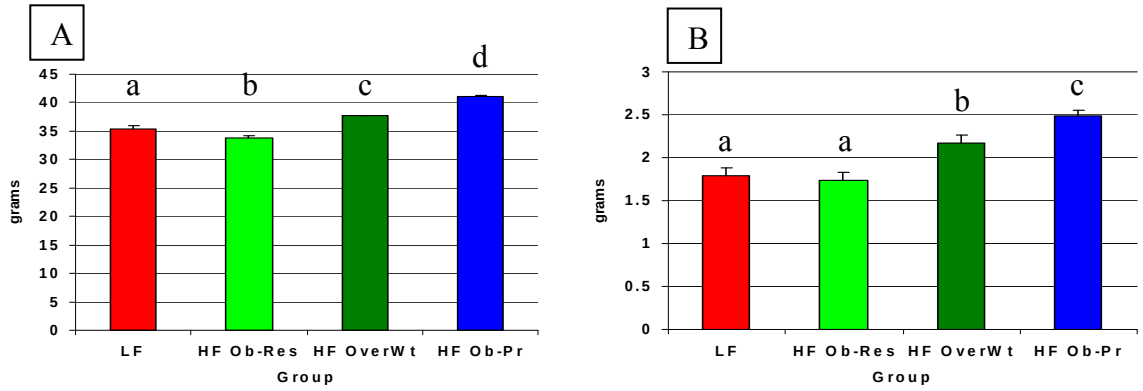


Figure 8. A. Body weights at 25 weeks of age and B. Fat pad weights at termination. Low-fat fed (LF), high-fat fed obesity-resistant (HF Ob-Res), high-fat fed overweight (HF OverWt) and high-fat fed obesity-prone (HF Ob-Pr). Bars represent means with standard error. Bars with different superscript letters are significantly different from each other.

Genitourinary tract and prostate cancer results: We found that the genitourinary tracts from the high-fat fed obesity-resistant mice were lowest (Table 3) and that the genitourinary tract weights from the high-fat fed obesity prone mice were highest. The low-fat fed mice and high-fat fed overweight mice did not have significantly different genitourinary tract weights. Because the TRAMP-C2 cells were injected subcutaneously in the flank we were able to harvest normal prostates from the mice to examine the effects of body weight and a high fat diet on normal prostates. We found that the high-fat fed obesity-prone mice had significantly heavier prostates as compared to Low-Fat and Obesity-Resistant groups (Table 3). The prostate weight was also higher in Obesity-Prone compared to Overweight mice but the difference was not significantly different.

Table 3. Genital-Urinary Tract and Prostate Weights in Male C57BL6 Mice with Diet-Induced Obesity (mean ± sem)

	Low-Fat	Obesity-Resistant	Overweight	Obesity-Prone
GU-Tract Weight (g) ANOVA p<0.0001	$0.51^{b} \pm 0.01$	$0.47^{c} \pm 0.01$	$0.51^{b} \pm 0.01$	$0.55^{a} \pm 0.01$
Prostate Weight (g) ANOVA p =0.005	$0.0668^{b} \pm 0.0021$ (n=19)	$0.0624^{b} \pm 0.0045$ (n=20)	$0.0721^{a,b} \pm 0.0029$ (n=19)	$0.08126^{a} \pm 0.0048$ (n=20)

Columns with different superscripts are significantly different from each other.

We examined the TRAMP-C2 tumors harvested from the mice and compared the different groups based on weight and volume. Figure 9A shows that the TRAMP-C2 tumors from the low-fat fed and high-fat fed obesity-prone mice were the lightest. The tumors from the high-fat fed obesity-resistant and the high-fat fed overweight mice were heavier. However, the differences were not significant. When the tumor volumes were computed the low-fat fed mice had the smallest tumors followed by the high-fat fed obesity-prone mice with the high-fat fed obesity-resistant and overweight mice having the largest tumors by volume but there was not a significant difference. When mice were divided into low-fat fed verses high-fat fed mice we found that the high-fat fed mice had heavier tumors than the low-fat fed mice (Fig 10A) ($P<0.01$). We also found that the average tumor volume of the high-fat fed mice was higher as compared to the low-fat fed mice (Fig 9B) ($P<0.0007$).

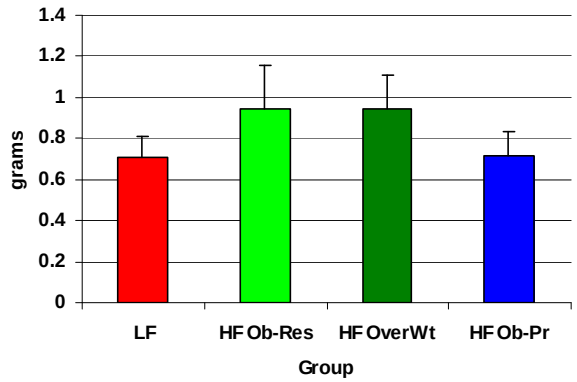


Figure 9A. Tumor weight at sacrifice. The four groups of mice are; low-fat fed (LF), high-fat fed obesity-resistant (HF Ob-Res), high-fat fed overweight (HF OverWt) and high-fat fed obesity-prone (HF Ob-Pr). Bars are means with standard error. ANOVA =NS

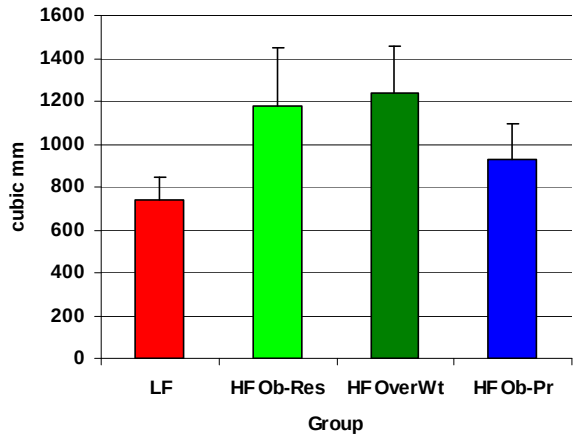


Figure 9B. Tumor volume at sacrifice. The four groups of mice are; low-fat fed (LF), high-fat fed obesity-resistant (HF Ob-Res), high-fat fed overweight (HF OverWt) and high-fat fed obesity-prone (HF Ob-Pr). Bars are means with standard error. ANOVA = NS

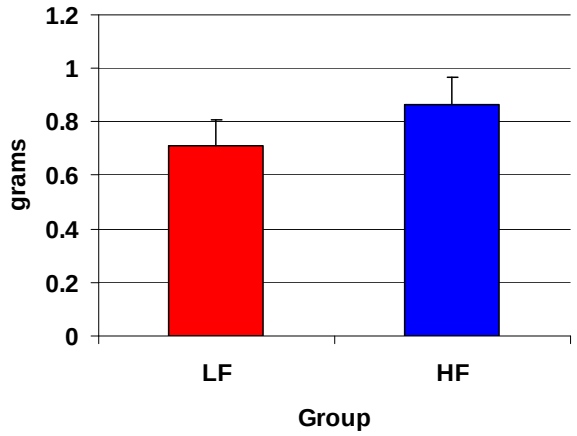


Figure 10A. Tumor weights at sacrifice. The average weight of the tumors from the low-fat fed mice (LF) is shown as red. The average weight of tumors from all of the high-fat fed mice (HF) is shown as blue. Bars are means with standard errors. Values were significantly different at $P<0.01$.

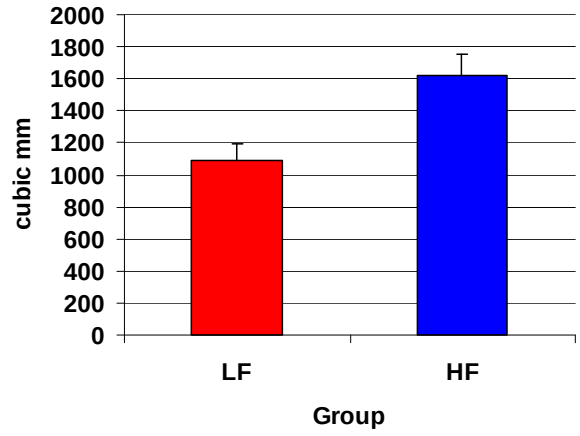


Figure 10B. Tumor volumes at sacrifice. The average tumor volume of the low-fat fed mice (LF) is shown as red. The average tumor volume of all of the high-fat fed mice (HF) is shown as blue. Bars are means with standard errors. Values were significantly different at $P<0.0007$.

Effect of Dietary-Induced Obesity on Prostate Cancer Development in TRAMP Mice:

While the above studies were underway we received funding from another source to determine the effect of diet-induced obesity in TRAMP mice. Some tissue analyses from these mice were conducted as part of this DOD grant. The protocol was based on earlier studies conducted in mice and rats that showed that high-fat fed animals could be separated by weight gain into groups that became obese, i.e., Obesity-Prone or stayed in the weight range of low-fat fed animals, i.e., Obesity-Resistant [43]. However in our studies unlike most others that discarded mid weight animals we included those as an Overweight group. When we utilized this protocol in MMTV-TGF-α female mice on C57BL6 background strain we found that mammary tumor development was shortened in proportion to the elevated body weight of the mice [35]. Since TRAMP mice are on the C57BL6 strain and male C57BL6 mice had previously been reported gain weight in a similar manner on a high-fat diet [39]. Mice were started on the experimental diets at 6 weeks of age and followed until 50 weeks of age or until disease burden necessitated euthanasia. There were 24 mice in each of the experimental groups.

Dietary –Induced Obesity Results: Mice were assigned to body weight groups based on weight gain from 6-18 weeks of age prior to when prostate tumor weight would impact body weights. As can be seen in Figure 11 final body weights of the Obese mice were higher than those of Low-Fat and Obesity-Resistant Mice. Fat pad weights are shown in Figure 12. Once again Obese mice tended to have the highest fat pad weights although as can be seen not all values were significantly different from each other.

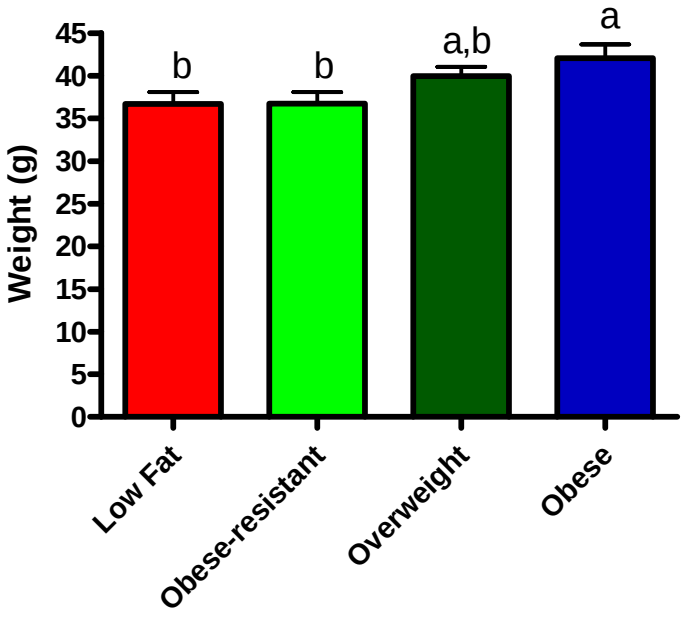


Figure 11. Final body weights of TRAMP mice a fed high fat diet. Bars are means with standard errors. Final body weight of the Obese mice was higher than that of Low-Fat and Obesity-Resistant mice. Body weight of Overweight mice was not significantly different from any other group. ANOVA P=0.0158 Bars with different superscript letters are significantly different from each other.

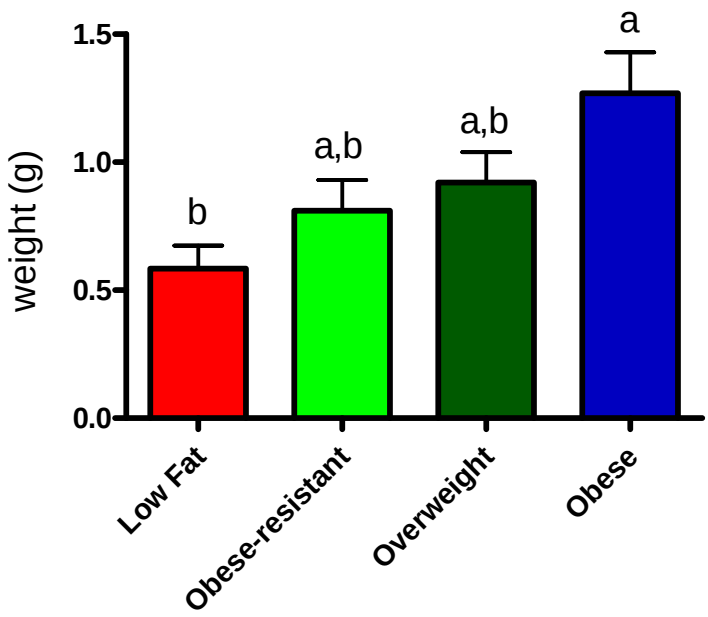


Figure 12. Fat pad weights of TRAMP mice fed high fat diets. Bars are means with standard errors. Obese mice had the heaviest fat pads. Low Fat mice had the lightest fat pad weight, although not significantly lower than Obesity-Resistant or Overweight mice. ANOVA $p<0.02$ Bars with different superscript letters are significantly different from each other.

As shown in Figure 13 there was no significant effect of dietary intervention on GU tract weight in the mice in the dietary-induced obesity experiment. In Figure 14 the classification of the prostate cancers found in the mice is shown. The Obese mice tended to have a higher percentage of poorly differentiated tumors compared to the three other groups. Low-fat fed mice did not have any poorly differentiated tumors.

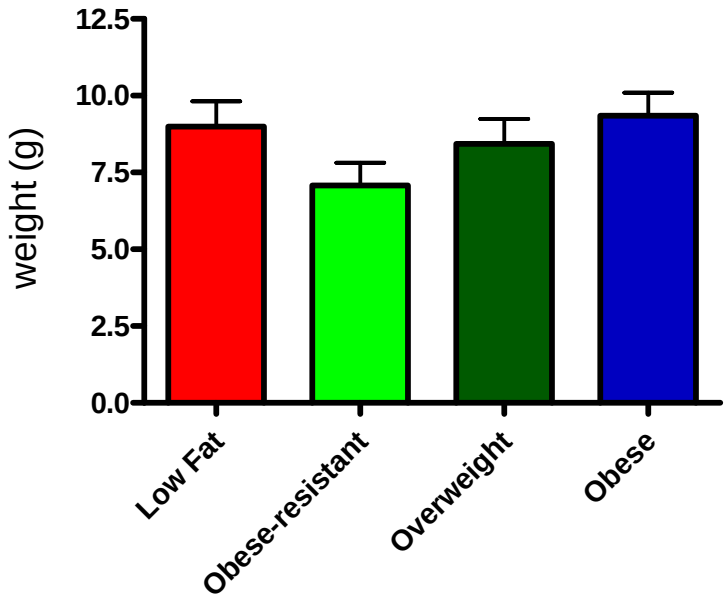


Figure 13. Genital-urinary (GU) tract weights of TRAMP mice fed a high fat diet. Bars are means with standard errors. Weights were not significantly different among the groups. ANOVA P=0.1729.

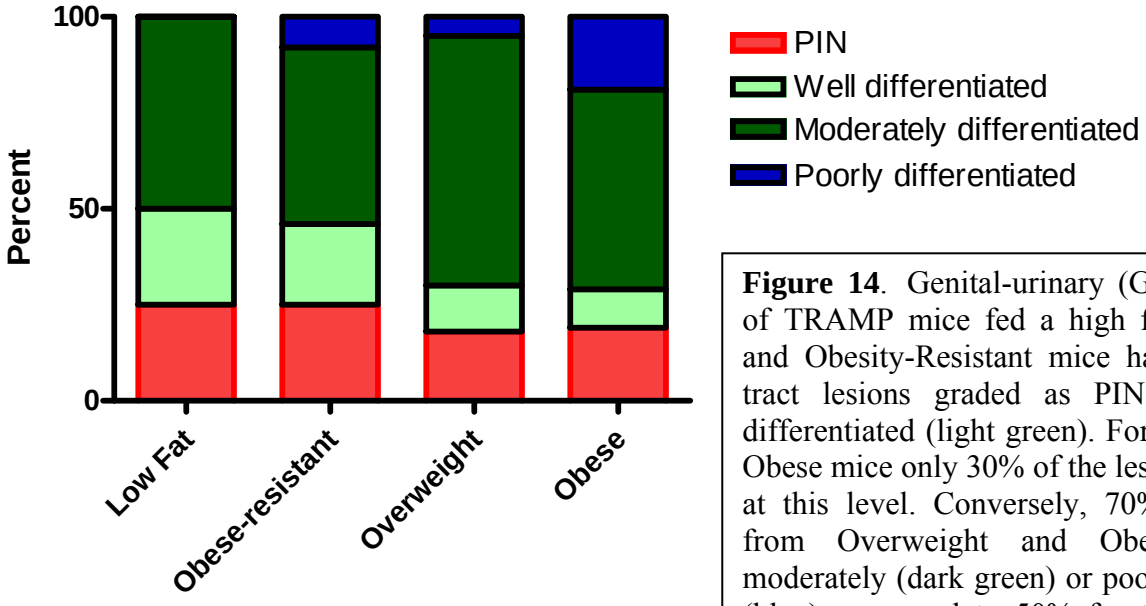


Figure 14. Genital-urinary (GU) tract weights of TRAMP mice fed a high fat diet. Low-Fat and Obesity-Resistant mice had ~50% of GU tract lesions graded as PIN (red) or well-differentiated (light green). For Overweight and Obese mice only 30% of the lesions were graded at this level. Conversely, 70% of the lesions from Overweight and Obese mice were moderately (dark green) or poorly differentiated (blue) compared to 50% for the Low Fat and Obese-Resistant mice. Metastases rates were similar for all groups, 63-74%, except for the Obese-Resistant group with a rate of 43%.

Synaptophysin expression is considered to be a reflection of neuroendocrine tumors (NE). This status was not affected by body weight classification of the mice fed the high fat diet. Therefore these results were combined for high fat diet mice and compared to the Low-Fat diet mice. The results are presented in Table 4. As can be seen NE tumors were found at a similar rate in the two groups. These tumors were detected at a

younger age and were all classified as high grade compared to NE negative tumors. In contrast only a percentage of NE negative tumors were classified as high grade. Interestingly, more high grade NE-negative tumors were found in mice fed the high fat diet. These findings were compared results from mice injected with GTG at 26 weeks of age. In those mice only 15% of the mice had NE-positive tumors. This was probably due to the fact that the mice first had to survive until 26 weeks of age to be enrolled in that study and mice tended to develop the NE tumors at a younger age as demonstrated in Table 4.

Table 4. NE positive prostate tumors in TRAMP mice fed high fat or low fat diets.

Group	Percent NE Positive	NE Status	Weight Increase 6-18 weeks	Final Body Weight	Age at Tumor Detection	Age at Death	GU Tract Weight	% High Grade Tumors
High Fat Diet Mice	35% (16/46)	Positive	10.23 +/- 0.75	36.6x +/- 1.64	26.0^{x} +/- 1.48	31.9^{x} +/- 2.52	8.49^{x} +/- 0.66	100^{x} (15/15)
		Negative	10.97 +/- 0.43	42.1 +/- 1.21	31.8 +/- 0.82	44.7 +/- 0.93	10.39 +/- 0.57	41 (11/27)
Low Fat Diet Mice	35% (6/17)	Positive	8.09 +/- 1.75	36.6 +/- 2.66	27.2^{x} +/- 2.91	35.3^{x} +/- 4.95	7.26^{x} +/- 1.17	100^{x} (6/6)
		Negative	7.97 +/- 0.73	39.7 +/- 1.66	31.7 +/1 1.22	45.8 +/1 1.58	10.41 +/- 1.03	20 (2/10)

Values are means ± standard errors. ^{x} indicates significant difference between positive and negative values.

Overall these findings do not produce a clear picture of the effect of body weight on prostate tumor development. There seems to be some indication that a high fat does impact tumor TRAMP-C2 growth. Interestingly, higher body weight was associated with an increase in GU-tract weight as well as prostate weight. How this would be related to prostate cancer development remains unclear. Future investigation may be to use a mouse model whereby prostate cancer cell lines are inoculated directly into the prostate gland. Data from the diet-induced protocol indicated little effect of body weight on age of tumor development and death but there did Obese mice did appear to be a greater percentage, 19% of poorly differentiated tumors compared to all other groups. The Overweight and Obesity-Resistant mice had 6% and 8% respectively, while there were none in the Low-Fat group. This finding suggests both a body weight and diet effect on tumor grade. A recently published study using a diet with a slightly higher fat content (42%) compared to our diet (33%) and with a high cholesterol content found that the diet had a much more negative impact on prostate cancer development than we have found [44]. Mice were started on the diets at 8 weeks of age and the study was terminated at 28 weeks of age. At the termination of the study 17% of the low fat mice had tumors compared to 33% of the Western type diet fed mice. Due to the many differences in the diet compositions as well as the study protocols it is difficult to make direct comparisons between this study and ours. Since eventually most TRAMP mice will develop prostate cancer the most that can be concluded is that the Western diet accelerated prostate cancer development.

KEY RESEARCH ACCOMPLISHMENTS:

1) Performed experiments as described to induce obesity at specific ages.
2) Preliminary interpretation indicates that obesity at a young age may be protective with respect to the development of prostate cancer. This is consistent with some human epidemiological studies.
3) Found that toxicity and mortality associated with GTG-induced obesity makes it impractical for continued use.
4) Determined that AdipoR1, AdipoR2 and Ob-Rb are expressed by TRAMP prostate cancer cells.
5) Measured serum estradiol and testosterone levels at various times over the course of the experiment but results were not consistent with any relationship to when tumors were detected.
6) We used an alternative approach to address the issue of the effect of body weight on prostate cancer development by feeding high fat diets to induce obesity. Body weights were affected by the diet. These mice were inoculated with TRAMP-C2 cells.
7) Mice with obesity had higher genital urinary tract weights as well as prostate weights.
8. Mice fed the high fat diet tended as a group to have higher tumor weights than the low-fat mice although this was primarily attributable to the obesity-resistant and overweight groups.
8) Performed in vitro experiments that indicate that TRAMP-C2 cell proliferation is inhibited by Acrp30 and that this effect is blocked by high levels of leptin.
9. Synaptophysin protein expression levels were determined in mice in the GTG experiment. There were enough surviving mice from those made obese at 26 weeks of age to do so.
10. Synaptophysin expression was also measured in mice from a diet-induced obesity experiment. There was no effect of body weight amongst the mice fed the high fat diet. Nor was there an effect of the high fat fed versus low fat fed mice.

REPORTABLE OUTCOMES:

ROLE OF OBESITY AT DIFFERENT AGES IN PROSTATE CANCER DEVELOPMENT IN TRAMP MICE
Margot P. Cleary, Melissa J.L. Bonorden, Olga P. Rogozina and Nancy K. Mizuno
Presented at the IMPACT meeting September 2007, Atlanta, GA. (abstract Appendix A)

CHARACTERIZATION OF ADIPONECTIN RECEPTOR EXPRESSION AND FUNCTION IN TRAMP PROSTATE TUMORS AND THE TRAMP-C2 CELL LINE
Michael E. Grossmann, Nancy K. Mizuno, Melissa J. L. Bonorden, Amitabha Ray and Margot P. Cleary
Presented at the Frontiers in Cancer Prevention Research meeting December 2007, Philadelphia, PA (abstract Appendix B)

IMPACT OF TWO TYPES OF OBESITY ON PROSTATE CANCER IN THE TRAMP MOUSE
Melissa J.L. Bonorden, Michael E. Grossmann, Olga P. Rogozina, D.Joshua Liao, Joseph P. Grande and Margot P. Cleary
Presented at the AACR meeting April 2009 in Denver, CO (abstract Appendix C)

ROLE OF THE ADIPONECTIN LEPTIN RATIO IN PROSTATE CANCER
Michael E. Grossmann, Nancy K. Mizuno, Melissa J.L. Bonorden, Amitabha Ray, Irina Sokolchik[2], Meena L. Narasimhan and Margot P. Cleary
Oncology Research 18:269-277,2009 (abstract Appendix D)

SYNAPTOPHYSIN EXPRESSION IN PROSTATE TUMORS FROM OBESE TRAMP MICE.
Michael E. Grossmann, M.J.L. Bonorden, D. Joshua Liao, Joseph P. Grande and Margot P. Cleary

Presented at the 2011 IMPaCT meeting March 2011, Orlando, FL. (abstract Appendix E)

CONCLUSIONS:
This has been a frustrating experience undertaking what we first thought would be a straight forward study using GTG to induce obesity. The study with the TRAMP-C-2 cells although easier to carry out did not provide as interesting results as what we expected. The diet-induced obesity study with TRAMP mice has provided some more interesting data which hopefully will provide the opportunity to design future experiments. However a better model which develops the disease more slowly and later in life would be really nice to make the findings more application to humans. We are currently working on a manuscript to present our results, i.e., GROWTH AND PROGRESSION OF TRAMP PROSTATE TUMORS IN RELATIONSHIP TO DIET AND OBESITY with the following coauthors Melissa J.L. Bonorden, Michael E. Grossmann, Sarah Ewing, Olga P. Rogozina, Amitbha Ray, Katai J. Nkhata, D.Joshua Liao, Joseph P. Grande and Margot P. Cleary.

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Appendix A

ROLE OF OBESITY AT DIFFERENT AGES IN PROSTATE CANCER DEVELOPMENT IN TRAMP MICE

Margot P. Cleary, Melissa J.L. Bonorden, Olga P. Rogozina, & Nancy K. Mizuno
Presented at Department of Defense Prostate Cancer Research Program Meeting- Innovative Minds in Prostate Cancer Today. Atlanta, GA September 2007

A number of epidemiological studies have implicated obesity as a risk factor for prostate cancer development. In addition, clinical and biochemical progression of prostate cancer has been reported to be shorter in obese men and mortality from prostate cancer is increased with elevated body weights[11]. Obesity was recently reported to be associated with higher prostate cancer grade at diagnosis, as well as with higher recurrence rates. The potential role of body weight in various aspects of prostate tumorigenesis is of interest given that the incidence of overweight/obesity is increasing throughout the world, and the potential for lifestyle changes to alter body weight status. The goal of the present study is to determine the effect of obesity induced at different ages on the development of prostate cancer using the TRAMP mouse model. Development of prostate cancer in the TRAMP mouse shares a number of similarities with the human disease. The experimental design was to induce obesity at three different ages and follow prostate cancer development. To attain this goal male TRAMP mice (C57BL6 background) were injected with gold-thioglucose (GTG) (0.5-0.8 mg/kg body weight in phosphate buffered saline (PBS)) at 6, 16 and 26 weeks of age. Mice were weighed weekly and palpated to detect prostate tumors. Mice were followed until 46 weeks of age or until disease burden necessitated euthanasia. Serial blood samples over the course of the study are also obtained. Control mice received injections of only the PBS vehicle. For the 26-week cohort 14 mice survived the GTG injection, of which 8 were obese (48.6 ± 2.6 grams). As expected some of the GTG mice did not develop obesity and were designated as non-Obese. Their body weight 39.1 ± 2.5 was in the body weight range of the PBS mice (36.7 ± 1.63). The final body weights for the Obese mice were significantly higher than for the other two groups (ANOVA $p < 0.05$). Fat pad weights followed a similar relationship. Total genital-urinary tract weights were not affected by body weight. Age of prostate tumor detection was not different among the three groups of mice (~33 weeks of age in age). Additionally age at death (~43 weeks of age) was similar among the groups. Serum and tissue analyses are presently being conducted. Additional cohorts of mice injected with GTG at 6 and 16 weeks of age are currently being followed. Overall it appears that GTG has a high toxicity and mortality rate in TRAMP mice (in contrast to our preliminary studies in C57BL6) mice. In the older mice induction of obesity had little effect on the development of prostate cancer. Ongoing studies will address the consequence of obesity on prostate cancer development in younger TRAMP mice.
Support:DOD PC050284 and the Hormel Foundation.

Appendix B

Characterization of Adiponectin Receptor Expression and Function in TRAMP Prostate Tumors and the TRAMP-C2 Cell Line

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Presented at the AACR Frontiers in Cancer Prevention Meeting- Philadelphia PA December 2007

Introduction: Obesity is associated with increased risk for more aggressive prostate cancer (Pca) as defined by an increase in the risk of Pca death and an increased chance of progression after surgery. Obesity may mediate its effects on Pca in part due to factors secreted from adipose tissue. One factor potentially involved in the interaction between Pca and obesity is adiponectin, also known as adipocyte complement-related protein of 30 kDa (Acrp30). Lower serum Acrp30 levels have been reported for Pca patients compared to patients with benign prostatic hyperplasia or healthy controls. In addition, lower expression levels of Acrp30 receptors are found in prostate tumors as compared to healthy prostate tissue. Here we assessed how Acrp30 impacted cell growth *in vitro* in the TRAMP-C2 cell line which is derived from a TRAMP prostate tumor and determined Acrp30 receptor expression in the TRAMP model.

Procedures: TRAMP-C2 cells (ATCC) were used in growth assays (CC8 kit Dojindo Laboratories). Whole cell extracts were obtained using Phosphosafe extraction reagent from Novagen for determination of adiponectin receptors (AdipoR1 and R2) and signaling proteins by western blot. Antibodies were from Santa Cruz Biotechnology except antibodies to AdipoR1 (Abcam Inc), AdipoR2 (Phoenix Pharmaceuticals, Inc.) and anti-rabbit secondary (Cell Signaling Inc.).TRAMP mice were euthanized at 50 weeks and urogenital tracts plus abnormal growths/tumors removed. Sections were stained with the rabbit ABC staining system for AdipoR1 and R2.

Results: There was a dose-related reduction in proliferation of the TRAMP-C2 cells after 48 hours in response to the addition of Acrp30. The difference in proliferation was statistically significant at physiological Acrp30 concentrations of 10 and 20 ug/ml (Student's t-test $p<0.03$ and 0.02 respectively) compared to untreated cells. Western blots indicated that AdipoR1 and AdipoR2 are both expressed by TRAMP-C2 cells. We also identified increases or decreases in phosphorylation of several growth associated signaling proteins with western blots. Acrp30 increased levels for both ERK1 and ERK2. The phosphorylation of Stat3 was decreased by the addition of fetal calf serum but this decrease was blocked by Acrp30.

We also found that tumors from TRAMP mice expressed the two receptors for Acrp30, AdipoR1 and AdipoR2. Using immunohistochemical analysis we found expression of AdipoR1 in prostate tumor tissue from TRAMP mice was mostly in epithelial cells on the apical membrane. AdipoR2 was present in the same areas as AdipoR1 but the staining was lower. Western blot analysis of frozen tissue from the same mice also indicated expression of AdipoR1 and AdipoR2 in prostate tumor tissue.

Conclusions: Here, we are the first to report the presence of AdipoR1 and AdipoR2 in prostate tumor tissues from TRAMP mice and in the TRAMP-C2 cell line which is derived from the prostate tumor of a TRAMP mouse. The receptors appear to be functional since proliferation of TRAMP-C2 cells was inhibited by addition of Acrp30. This decrease in cell growth may be attributable to increased signaling through ERK 1/2 since Acrp30 increased the phosphorylation of ERK 1/2. We are currently investigating the levels of Acrp30 *in vivo* with ongoing mouse studies in relationship to body weight and Pca development. Support from DOD PC 050284 and The Hormel Foundation.

Appendix C.

Impact of Two Types of Obesity on Prostate Cancer in the TRAMP Mouse
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Presented at AACR meeting Denver, CO April 2009

Epidemiological studies suggest that body weight plays a role in prostate cancer development and obesity is associated with higher cancer grade and greater recurrence and mortality rates. To clarify these issues two studies were undertaken using the TRAMP mouse model of prostate cancer. First obesity was induced at 6, 16 and 26 wk of age by injection of gold-thioglucose (GTG) (0.5-0.8 mg/kg body weight in phosphate buffered saline (PBS). Mice were weighed and palpated weekly to detect prostate tumors until 46 wk of age or until disease burden necessitated euthanasia. Overall GTG had high toxicity and mortality rates in TRAMP mice (in contrast to preliminary studies in wildtype C57BL6 mice) resulting in limited data from the 6- and 26-wk cohorts. For the 26-wk cohort 14 mice survived the GTG injection, of which 8 were obese (48.6 ± 2.6 grams) (ANOVA $p < 0.05$) compared to the non-Obese GTG mice with a body weight of 39.1 ± 2.5 grams, which was similar to PBS injected mice (36.7 ± 1.63). Fat pad weights had a similar relationship. Genital-urinary tract (GUT) weights were not affected by body weight. Age of prostate tumor detection (~33 wk) or death (~43 wk) was not different among the groups. Similar results were obtained for the 6-wk cohort despite their much longer exposure to obesity. In general GTG-Obese mice had lower metastases rates although GUT pathology was similar to lean mice. In the second study TRAMP mice were fed a moderately high fat (33% fat calories) diet from 6 wk of age. Based on body weight gain from 6-18 wk of age mice were divided into Obesity-Prone, Overweight and Obesity-Resistant groups (n=24). A Low-Fat group (n=24) was included for comparison to the Obesity-Resistant mice. Due to low body weights mice that died prior to 30 wk of age were removed from calculations resulting in final numbers ranging from 16-21/group. Final body weights of Obesity-Prone mice were significantly heavier than Obesity-Resistant mice. Fat pad weights of Obesity-Prone mice were significantly heavier than all other groups. When normalized to body weight, fat pad weights of Obesity-Prone mice were significantly greater than those of Low-Fat mice. There were no significant effects of body weight or diet on GUT weight or GUT relative to body weight among the groups. There were no differences in age to tumor detection (29-32 wk) or death (37-41 wk) among the groups. Metastases rates (63-73%) were similar for all groups except for the Obesity-Resistant mice which had a rate of 43%. There was a trend for Obesity-Prone and Overweight mice to have lower incidence of PIN and higher incidence of moderately to poorly differentiated prostate tissues compared to the Obesity-Resistant and Low-Fat mice. These findings are consistent with epidemiological evidence indicating obesity's role in prostate cancer is associated with more aggressive disease. Diet-induced obesity provided a better obesity related model than did GTG in TRAMP mice. (Support AICR, DOD and The Hormel Foundation).

Appendix D

Oncology Research, Vol. 18, pp. 269–277

0965-0407/09 $90.00 + .00
DOI: 10.3727/096504009X12596189659367
E-ISSN 1555-3906
www.cognizantcommunication.com

Role of the Adiponectin Leptin Ratio in Prostate Cancer

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We hypothesize that adiponectin and leptin may be capable of mediating some of the effects that body weight has on prostate cancer and that a mouse model may be effective to examine this hypothesis. We found that tumors from the TRAMP prostate cancer model expressed adiponectin and leptin receptors. TRAMP-C2 prostate cancer cell proliferation was reduced by adiponectin. Leptin was able to block the ability of adiponectin to reduce cell proliferation through altered signaling of the ERK pathway. Overall, this work suggests that adiponectin, leptin, and their receptors may play an important role in prostate cancer.

ACKNOWLEDGMENTS: Support for this work was provided by grants from the Department of Defense (PC020457 and PC050284) from the American Institute for Cancer Research (AICR) and the National Science Foundation Award No. 0350439 as well as by The Hormel Foundation.

Appendix E

SYNAPTOPHYSIN EXPRESSION IN PROSTATE TUMORS FROM OBESE TRAMP MICE.

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Presented at the 2011 IMPaCT meeting March 2011, Orlando, FL.

Background: Obesity may play a role in prostate tumor aggressiveness, but other factors (disease stage, diet, and gene expression) may affect data interpretation. We evaluated two types of obesity on prostate tumorigensis in TRAMP/C57BL6 mice and assessed synaptophysin (SN) expression in prostate tumors as an indicator of the presence of neuroendocrine tumors. Methodology: In Study 1 obesity was induced at 26 wk of age by ip injection of goldthioglucose (GTG) (0.8 mg/kg bw in PBS). In Study 2 TRAMP mice were fed a 33% fat calorie diet from 6 wk of age. Based on weight gain from 6-18 wk of age mice were divided into Obesity-Prone, Overweight and Obesity-Resistant groups. A Low-Fat group was included for comparison (n=16-24/groups). Mice were palpated to detect tumors until 46 (Study 1) or 50 wk of age (Study 2) or until disease burden necessitated euthanasia. SN expression in prostate tumors was determined by western blot. Results: In Study 1 GTG-TRAMP mice had poor survival. Of the TRAMP mice given GTG 8/14 surviving mice were obese; 48.6±2.6 g (ANOVA $p < 0.05$) compared to 39.1±2.5 g for non-Obese GTG and 36.7±1.63 for PBS mice. Genital-urinary tract (GUT) weights were not affected by body weight. Ages of prostate tumor detection (~33 wk) or death (~43 wk) were not different. GTG-Obese mice tended to have fewer poorly differentiated tumors and lower metastases compared to lean mice. No SN+ tumors were detected in GTG mice regardless of body weight while for PBS mice 30% of the tumors were SN+. In Study 2 terminal body weights of Obesity-Prone mice were significantly heavier than Obesity-Resistant mice while fat pad weights of Obesity-Prone mice were significantly heavier than all other groups. There were no effects of body weight or diet on GUT weight. There were no differences in age to tumor detection (29-32 wk) or death (37-41 wk) among groups. Metastases rates (63-73%) were similar except for the Obesity-Resistant mice, 43%. Obesity-Prone mice had 0% poorly differentiated tumors compared to 9%, 8% and 19% for Overweight, Obesity-Resistant and Low-Fat groups respectively. SN+ tumors were identified in 37%, 21%, 46% and 35% of these groups. SN+ tumors were detected earlier (25-27 vs 29-34 wks of age) than SN- tumors and mice with SN+ tumors died at younger ages (30-35 vs 42-46 wks of age) than those with SN- tumors. SN+ tumors were 40-140% heavier in mice fed the high-fat diet than SN- tumors but weighed the same in low-fat diet mice. Conclusions: A moderately high-fat diet provided an obesity model in TRAMP mice. Obese mice had fewer aggressive tumors. Obesity-Prone and Overweight mice with SN+ tumors died at a younger age than Obesity-Resistant mice with SN+ tumors indicating a complex relationship for diet and body weight on prostate cancer. Impact: Epidemiological studies investigating the effects of obesity on prostate cancer should take into consideration the neuroendocrine status of the tumors.